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RESEARCH ARTICLE

Sticklebacks adapted to divergent osmotic environments show differences in plasticity for kidney morphology and candidate gene expression

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ABSTRACT

Novel physiological challenges in different environments can promote the evolution of divergent phenotypes, either through plastic or genetic changes. Environmental salinity serves as a key barrier to the distribution of nearly all aquatic organisms, and species diversification is likely to be enabled by adaptation to alternative osmotic environments. The threespine stickleback (*Gasterosteus aculeatus*) is a euryhaline species with populations found both in marine and freshwater environments. It has evolved both highly plastic and locally adapted phenotypes due to salinity-derived selection, but the physiological and genetic basis of adaptation to salinity is not fully understood. We integrated comparative cellular morphology of the kidney, a key organ for osmoregulation, and candidate gene expression to explore the underpinnings of evolved variation in osmotic plasticity within two populations of sticklebacks from distinct salinity zones in the Baltic Sea: the high salinity Kattegat, representative of the ancestral marine habitat; and the low salinity Bay of Bothnia. A common-garden experiment revealed that kidney morphology in the ancestral high-salinity population had a highly plastic response to salinity conditions whereas this plastic response was reduced in the low-salinity population. Candidate gene expression in kidney tissue revealed a similar pattern of population-specific differences, with a higher degree of plasticity in the native high-salinity population. Together these results suggest that renal cellular morphology has become canalized to low salinity, and that these structural differences may have functional implications for osmoregulation.

KEY WORDS: Osmoregulation, Local adaptation, Adaptive plasticity, Kidney morphology

INTRODUCTION

Widely distributed species frequently face differing and localized environmental challenges. Plasticity in physiological function may help to facilitate acclimatization to and/or colonization of novel environments whereas inter-individual variability and associated

bioenergetic costs and trade-offs may ultimately lead to adaptive evolution (Watt, 1985; Schulte, 2001; Dalziel et al., 2009). However, complete understanding of divergent evolutionary trajectories in response to the dominant abiotic factors experienced by such cosmopolitan species remains a central challenge in biology, requiring integration of analyses at all levels, from molecules within cells to whole-organism performance in relation to the broad range of conditions comprising a species' physiological niche (Pough, 1989). For aquatic organisms, the maintenance of plasma ion concentrations represents a unique set of physiological challenges. For example, freshwater species inhabit a hypotonic environment where they tend to gain water and lose salts via exposed membranes. In contrast, marine species live in a hypertonic environment in which salts are gained and water lost to the external environment. Osmoregulation under both conditions is energetically costly and necessitates active ion transport against a concentration gradient employing a variety of molecular pumps and channels (Perry and Fryer, 1997). In fishes, these physiological mechanisms are coordinated between gills, intestines and kidneys (Eddy and Handy, 2012). Specifically, ionic balance is maintained in seawater through a combination of branchial and renal excretion of salts, and oral ingestion and intestinal uptake of water (Evans, 2008). The opposite occurs in hypotonic environments such as weakly brackish or freshwater: salts are reabsorbed across the gills and intestines whereas excess water is filtered by the highly vascularized kidney (Hickman and Trump, 1969; Perry and Fryer, 1997; Hentschel et al., 2000). These specific mechanisms have been studied in great detail in the gill (Evans et al., 1999, 2005; Hwang et al., 2011) and intestine (Lahlou, 1983; Grosell, 2006) of marine and freshwater fishes, and have revealed various degrees of plasticity in response to salinity transfer. However, the extent of associated cellular remodeling and/or transcriptional changes in the kidney has received less attention (but see Perry et al., 2003; Wang et al., 2014), especially in euryhaline species that can tolerate a wide range of salinities.

The main functions of the kidney include filtering wastes, absorbing nutrients and water, and maintaining body fluid concentrations (Eaton, 2012). These are achieved by the nephron, the functional unit of the kidney, which is composed of several structural subunits. Chief among these are the blood-filtering glomeruli, which mark the beginning/head of nephrons, and the connected tubules, which ultimately discharge urea and water via the collecting duct. The tubular epithelium can be further distinguished into distinct proximal and distal segments, which in turn have unique roles in modifying the salt and water composition of urine via specific solute transport activities (Hickman and Trump, 1969; Reimschuessel, 2001). These kidney structures are also known to vary both within and among species, depending on their

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List of abbreviations

ACTG1	actin γ 1
AQP3A	aquaporin 3a
ATP1A1A.4	Na ⁺ /K ⁺ -transporting ATPase, α 1a polypeptide, tandem duplicate 4 (i.e. <i>ATP1A1</i>)
CD2AP	CD2-associated protein
KIRREL3L	kin of IRRE-like 3-like (i.e. <i>NEPH3</i>)
SLC9A3.2	solute carrier family 9 (sodium/hydrogen exchanger), member 3, tandem duplicate 2 (i.e. <i>NHE3B</i>)
SLC12A1	solute carrier family 12, member 1 (i.e. <i>NKCC2</i>)
DIC	deviance information criterion
G×E	genotype-by-environment interaction
KAS(L)	Kaskinen–low-salinity population
MAR(H)	Mariager Fjord–high-salinity population
PDI	posterior density interval
ppt	parts per thousand (‰)
V _G	genetic variance (broad-sense)

local habitats (Hickman and Trump, 1969). For example, nephrons of freshwater fish typically have large, well vascularized glomeruli and tubules to retain salts and produce dilute urine for elimination of excess water (Hickman and Trump, 1969). In contrast, marine fishes lose water to the external environment; therefore, they tend to have poorly developed glomeruli and lower glomerular filtration rates to maximize water retention (Reimschuessel, 2001). In some cases, certain marine and estuarine species are entirely devoid of glomeruli and/or distal tubules (Hickman and Trump, 1969; Reimschuessel, 2001). In general, cellular form may influence not only immediate renal function, but ultimately the type of environment to which an animal may acclimatize. Hence, studying aspects of renal function in these divergent anatomical structures can provide insights to their potential role in local adaptation.

The threespine stickleback (*Gasterosteus aculeatus* Linnaeus 1758) has emerged as a premier model in the study of adaptive evolution (Bell and Foster, 1994; Gibson, 2005; Jones et al., 2012). A marine species in origin, the ancestral phenotype has been highly conserved since the Miocene Epoch; however, repeated colonization of freshwater throughout the species' global distribution has resulted in pronounced evolutionary changes. Given the species' capacity to acclimatize and adapt to a wide range of salinities – its distribution extends throughout coastal marine and lowland freshwaters of the boreal and temperate northern hemisphere (Bell and Foster, 1994) – it is an ideal model for studies of kidney structure and function. Whilst the kidney has been the target of many physiological studies aimed at understanding the mechanisms of ion transport in various species of marine and freshwater fishes (Yancheva et al., 2016), it has received considerably less attention in sticklebacks. Earlier comparative studies revealed that the renal structures of freshwater sticklebacks are better developed than those from the marine environment (Ogawa, 1968; Wendelaar Bonga, 1973). Likewise, upon freshwater transfer, the kidneys of anadromous sticklebacks undergo structural modifications, acquiring characteristics of freshwater fish, suggesting a high degree of renal plasticity in marine/anadromous sticklebacks (Hickman and Trump, 1969; Wendelaar Bonga, 1973). Interestingly, no studies to date have explored whether this morphological plasticity is maintained in sticklebacks adapted to freshwater or weakly brackish environments. Moreover, whereas studies at the functional level have described transcriptional responses to salinity in both marine/anadromous and freshwater sticklebacks (McCairns and Bernatchez, 2010; Judd, 2012; Taugbøl et al., 2014; Divino et al., 2016), most have focused on gill rather

than kidney tissue. Although a recent transcriptome-wide assay of salinity-induced changes in stickleback kidneys has provided a fresh insight into this important model system (Wang et al., 2014), its use of wild-caught test fish limits the ability to unambiguously discern genetically based divergence in expression from confounding environmental factors.

Locally adapted populations of sticklebacks in the Baltic Sea represent an ideal system to differentiate genetic from environmental sources of variation in kidney morphology and function. The Baltic Sea is characterized by a pronounced east–west and north–south salinity gradient (Fig. S1), with its northern arm, the Gulf of Bothnia, being almost freshwater (Olenin and Leppäkoski, 1999), and the Danish Straits being nearly marine (20 ppt). The threespine stickleback is one of the most abundant fishes of the Baltic Sea, and fine-scale population structuring in this area appears to be associated with existing salinity gradients (DeFaveri et al., 2013; Guo et al., 2015). In the face of such environmental variation, it is expected that populations from different environments would have a wide variation in kidney morphology, ranging from highly developed kidney structures in populations residing in areas of low salinity to poorly developed kidneys in those native to high salinity (Reimschuessel, 2001). Fitness-based tests of whole-organism response to reciprocal salinities have established that populations are indeed adapted to local conditions (DeFaveri and Merilä, 2014); likewise, marker-based tests of selection in candidate genes associated with osmoregulation have also been suggestive of adaptive divergence to the different salinity regimes across the Baltic Sea (Shimada et al., 2011; DeFaveri et al., 2013). For example, divergence at the *NHE3* locus, a solute carrier that has an important role in osmoregulatory processes (Evans, 2008), was found to be three times higher than in neutral markers when comparing populations from the Danish Straits and the Bay of Bothnia ($F_{ST}=0.033$ versus 0.011; data from DeFaveri et al., 2013). Similar divergence was also seen in loci associated with ion transport (*ATP1A1*; $F_{ST}=0.055$) and water channel proteins (*AQP3A*; $F_{ST}=0.032$). Whether genetic divergence in candidate genes is also accompanied by differentiation in their expression remains a question in need of further study. It is, however, expected that divergent populations will have evolved differential expression in many osmoregulatory candidate genes (McCairns and Bernatchez, 2010; Whitehead et al., 2013). For example, ion transporters and co-transporters such as *ATP1A1* and *NKCC* are expected to show increased expression in high-salinity conditions whereas Na⁺/H⁺ exchangers and water channel proteins like *NHE3B* and *AQP3A* are expected to have higher transcriptional levels in low salinity. Yet, given the euryhaline nature of the species, such changes might also be reflected in their inherent physiological plasticity (McCairns and Bernatchez, 2010; Whitehead et al., 2013). Nevertheless, limits to plasticity are almost certainly imposed by energetic costs and trade-offs (Van Tienderen, 1997; DeWitt et al., 1998; Van Kleunen and Fischer, 2005), which may differ among populations of a given species (DeWitt et al., 1998). Thus, stickleback populations from divergent osmotic conditions may also serve as ideal models to determine the relative role of plasticity during the process of adaptation, and whether this plasticity differs between populations that are adapted to different salinities.

In this study, we compared the difference in renal morphology and inferred renal function between two stickleback populations adapted to different salinity conditions in the Baltic Sea area (viz. high salinity Danish Strait, 20 ppt; low salinity Bay of Bothnia, 5 ppt). After rearing multiple families from each population under native and reciprocal salinity conditions, we quantified gross-scale

kidney cell morphology and mRNA abundance in candidate osmoregulatory genes in order to document plastic (i.e. purely environmentally mediated) responses to salinity in contrast to genetically based divergence (including genetic differences in plasticity, i.e. genotype-by-environment interactions) that have evolved between these populations. Three candidate genes were selected based on their known osmoregulatory function as important ion-transporting and water channel proteins (*ATP1A1*, *AQP3A* and *NHE3B*), which have been shown to play integral roles in maintaining osmotic and ionic balance across key osmoregulatory organs, including the kidney (Cutler and Cramb, 2001; Perry et al., 2003; Finn and Cerdà, 2011). Additionally, previous work cataloging divergence among ecologically diverse stickleback populations in the Baltic Sea identified these genes as putatively selected outliers based on their genomic divergence between freshwater and marine populations within the study region (DeFaveri et al., 2011, 2013; Shimada et al., 2011). Three additional candidate genes (*NKCC2*, *CD2AP* and *NEPH3*) were selected based on their known functional role in glomerular filtration (Shih et al., 1999; Lehtonen et al., 2000; Hentschel et al., 2007), and whose differential expression in relation to salinity has been demonstrated in glomerular podocytes and tubules of various fish species (Cutler and Cramb, 2001; Nishimura et al., 2004).

MATERIALS AND METHODS

Fish sampling, breeding and maintenance

Adult sticklebacks were sampled during the breeding season (May–June) of 2011 from two demes inhabiting different salinity conditions in the Baltic Sea: Mariager Fjord, Denmark (MAR; 56°38'58"N, 09°57'05"E, see also Fig. S1), a region of high salinity (20 ppt mean annual salinity; range 17–24 ppt); and the Gulf of Bothnia, near the municipality of Kaskinen, Finland (KAS; 62°23'02"N, 21°13'30"E, see also Fig. S1), a region of low salinity (3–5 ppt mean annual salinity). In the text, population codes are followed by (H) and (L) – denoting high and low salinity, respectively – to highlight the environmental status of each. While neutral genetic divergence among these two populations is roughly one per cent, divergence in candidate gene-based markers with putative osmoregulatory roles is five times higher (data from DeFaveri et al., 2013). Differential responses to salinity treatment have also been documented in these specific populations, notably a reduction in fitness (i.e. larval survival, juvenile growth) in fish native to the low-salinity region (KAS) reared at high salinity (DeFaveri and Merilä, 2014). Ten 'purebred' F1 families were established within in each population; see DeFaveri and Merilä (2014) for details on crossing and rearing. Briefly, each family was divided and individuals from each family were reared in either of two salinity treatments (i.e. 5 and 20 ppt), chosen to reflect the local salinities at each of the two sampling sites to which source populations are adapted. Fish were maintained in this full factorial design for one year, at which time eight families were randomly selected per treatment. Although sticklebacks can come into reproductive condition at this age, we were careful to select individuals that were not showing any visible signs of sexual maturity (i.e. no nuptial coloration or gravidity) and no discernible gonads during dissections (i.e. most probably immature males). This is particularly important because the kidneys of males undergo major structural and functional changes during breeding (De Ruiter and Wendelaar Bonga, 1985); however, this is unlikely to affect the current results as only immature fish were used. In total, five individuals per family were sampled for gene expression analysis ($N=160$). Due to logistic constraints only three families per

treatment were used for morphological analysis, from which two individuals per family and treatment were sampled ($N=24$). Immediately after euthanization by decapitation, the kidney was quickly dissected from the body and preserved either in 10% formalin (if used for kidney tissue morphology analysis) or flash-frozen in liquid nitrogen and stored at -80°C (if used in gene expression analysis). Wild fish were collected in accordance with national fishery regulations, and experiments were conducted under a license from the National Animal Experiment Board of Finland.

Gross-scale cellular morphology of kidneys

Following tissue fixation, kidneys were embedded in paraffin. Several 5 μm cross-sections were made with a microtome throughout the entire length of each kidney sample and stained (haematoxylin and eosin; Fischer et al., 2008) for visualization and characterization of the different cell types (namely glomeruli and tubules). Kidney sections were examined under a Nikon Eclipse 800 light microscope (Nikon Nordic, Vantaa, Finland), and a digital photograph of each was taken with a Spot Image digital camera at $\times 40$ magnification (Olympus BX61, Olympus Finland, Espoo, Finland). Measurements were taken from digital photographs using IMAGEJ (Abramoff et al., 2004). As we could not identify distal and proximal ends, nine cross-sections throughout the length of each kidney were chosen to capture within-kidney variation in structural components. From each section, five tubules were chosen randomly and measured for total tubule diameter, lumen diameter and cell height (Fig. S2). Due to the limited numbers of available glomeruli, only five from each sample were measured. In all cases, each structural element was measured eight times from differing starting points around the entire circumference of the structure – in this way we captured elements of both intra-structural variation and measurement area in subsequent analyses.

Candidate gene selection and primer design

As detailed in the Introduction, candidate genes were selected based on their osmoregulatory function as important ion-transporting and water channel proteins (*ATP1A1*, *AQP3A* and *NHE3B*), as well as their functional role in glomerular filtration (*NKCC2*, *CD2AP* and *NEPH3*). A summary of the putative osmoregulatory function of each candidate gene is presented in Table 1. Actin (*ACTG1*) was used as a reference gene for normalizing transcription because its expression in the kidney does not change in response to environmental salinity (Lu et al., 2011). Primers for each gene were designed based on their respective cDNA sequences reported in the stickleback genome (Ensembl Genome Browser, database version 78.1; accessed July 2014). For each gene, sequential exon pairs were exported to Primer3 software (Untergasser et al., 2012) to design unique primers that spanned the exon–exon boundary, to avoid amplification of any residual genomic DNA. Predicted primers were BLAST searched against the stickleback genome to ensure the uniqueness of the targeted transcript. Details of the primer sequences are given as supplementary material (Table S1).

RNA extraction, cDNA synthesis and quantification of candidate gene expression

Total RNA was extracted from kidney tissue using an RNeasy Mini Kit, following the manufacturer's instructions (Qiagen, GmbH–Germany). Initial RNA concentration was determined by using a QubitTM assay kit, according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA), and then standardized to equimolar concentration ($84\text{ ng }\mu\text{l}^{-1}$) before cDNA synthesis. Twelve μl of total RNA from each sample was reverse transcribed

Table 1. Putative osmoregulatory function of candidate genes

Candidate gene	Putative osmoregulatory function
<i>AQP3A</i>	Water channel protein involved in maintaining osmotic balance; expressed in kidney tubules (Cutler et al., 2007; Engelund and Madsen, 2011). Outlier between freshwater and marine stickleback populations (DeFaveri et al., 2011; Shimada et al., 2011).
<i>ATP1A1</i>	Active ion transport across plasma membranes; expressed in both gill and kidney tissues during osmoregulation (Perry et al., 2003; Wang et al., 2014). Outlier between freshwater and marine stickleback populations (DeFaveri et al., 2011; Shimada et al., 2011).
<i>CD2AP</i>	Maintenance of filtration by glomerulus; expressed in both glomeruli and tubules (Lehtonen et al., 2000; Li et al., 2000; Hentschel et al., 2007).
<i>NEPH3</i>	Glomerulus development and filtration; expressed in glomerulus and tubules (Wang et al., 2012; Ristola and Lehtonen, 2014).
<i>NHE3B</i>	Na ⁺ ion regulation in low salinity; expressed in gills and in mammalian kidneys – renal osmoregulation in teleosts hypothesized (Cutler and Cramb, 2001; Yan et al., 2007; Chang et al., 2013). Outlier between freshwater and marine stickleback populations (Shimada et al., 2011; DeFaveri et al., 2013).
<i>NKCC2</i>	Freshwater osmoregulation – dilution of urine to conserve ions; expressed in kidney tubules (Kato et al., 2011; Wang et al., 2014).

Note that for sake of brevity, other putative functions beyond maintenance of homeostasis have not been listed. All genes selected have been shown to be expressed in the kidneys of at least one other teleost species.

to cDNA using the QuantiTect Reverse Transcription Kit, according to the manufacturer's instructions (Qiagen, GmbH–Germany).

Candidate gene expression was inferred by relative quantification of mRNA, measured with quantitative PCR (qPCR) using a Bio-Rad PCR system. Each qPCR contained 2 µl of diluted (1:10) cDNA, 3 µl of primers (150 nmol l⁻¹ each) and 5 µl of PCR master mix (Bio-Rad, iQTMSYBER[®] Green Supermix), in a final reaction volume of 10 µl. PCRs were run on 384-well plates containing two cDNA samples from each individual, with one sample used for amplification of a given candidate gene, and the other used for the reference gene (*ACTG1*); a reference individual (positive control) and a negative control (water+PCR reagents) were also included in all plates. In total, three technical replicates were run for each candidate gene on three separate plates.

Raw amplification data were used to calculate initial mRNA concentration (N_0) for each PCR with the LinRegPCR software (Čikoš et al., 2007). Following baseline correction, a window-of-linearity was used to perform regression analysis of the fluorescence data from the exponential phase. To capture experimental error and random variation among runs/plates, each N_0 value for a candidate gene was normalized against N_0 values of the reference gene in all plates corresponding to that given candidate gene's reaction. For example, the technical replicate on plate 1 was normalized against the reference replicates on plates 1, 2 and 3, likewise for technical replicates 2 and 3. The final data set consisted of nine pseudo-replicate normalized values per individual.

Data analysis

All data analysis was performed in the R computing language (R Foundation for Statistical Computing; <https://www.r-project.org>). Both kidney cell measurements and gene expression data were analysed via general mixed-effects models employed in the 'MCMCglmm' package (Hadfield, 2010). For both data sets (i.e.

kidney structure and mRNA expression), population of origin (here used as a proxy for locally adapted genotypes; G), rearing environment (E) and genotype-by-environment interaction (G×E) were assigned as fixed effects; however, random effects differed between data sets. For kidney data, random effects included variation among full-sibling families, variation among longitudinal sections, and repeated measures of each structure (herein termed measurement error, V_{meas}). For mRNA data, random effects included variation among families, variation due to repeated measures and variation among PCR plates, nested within the suite of repeated measures of a given individual (V_{plate}) – for the latter, three pseudo-replicate values (calculated from each reference gene) were generated for each plate containing a given technical replicate. For kidney cell morphology data, models were run for 200,000 iterations, with a burn-in of 100,000 and a thinning interval of 100 to yield 1000 posterior estimates from each sampling chain; for gene expression data, these parameters were 2,000,000, 1,000,000 and 1000, respectively. In both cases, default priors were used, and model convergence was verified by visual inspection of trace, density and autocorrelation plots. Significance of fixed effects was profiled from the posterior distributions of model coefficients. For plotting, we estimated/predicted fixed-effects means conditioned on random effects.

For each trait, three models were tested: (i) a minimal/null model for which random effects only included technical variation (e.g. kidney section, PCR plate) and repeated measurement; (ii) a model including broad-sense genetic variation (i.e. including all sources of genetic variance, as opposed to a decomposed expression of only additive genetic variance) for trait expression, modeled as among-family variation in model intercepts; and (iii) maximal model, wherein random effects also included a family-specific environmental response (i.e. slope, $\beta_{\text{Treat|Fam}}$) as a proxy for broad-sense genetic variation in plasticity. Model selection was based on the deviance information criterion (DIC).

RESULTS

Kidney cell morphology

Nephron morphology responded in a population-specific manner to the effects of environmental salinity. In both populations, glomerulus diameter was significantly reduced in the high-salinity treatment (20 ppt; Fig. 1; Table 2 – representative histological images can be seen in Fig. S3). This effect was most pronounced in the native high-salinity population MAR(H), whose mean glomerulus diameter was reduced by 31.5% compared with a 15.6% reduction observed in the low-salinity population KAS(L). This led to a significant difference between populations in the high-salinity treatment, where mean glomerulus diameter was 20.6% smaller in MAR(H) compared with KAS(L) individuals ($P=0.026$; Table 2). In the low-salinity treatment (5 ppt), mean glomerulus diameter did not differ significantly between populations ($P=0.706$; Table 2).

None of the tubule measurements differed between salinity treatments in the KAS(L) population (overall tubule diameter, $P=0.984$; lumen diameter, $P=0.062$, Table 2). In contrast, MAR(H) individuals exhibited significantly smaller tubules (overall tubule diameter: 19.9%, $P<0.001$; lumen diameter: 28.6%, $P<0.001$) when reared at 20 ppt (Table 2; Fig. 2A,B; see Fig. S4 for representative sections). Mean tubule cell height did not differ between populations, nor was it affected by salinity (Table 2); however, variance appears to have increased for individuals reared at 20 ppt (Fig. 2C).

Inclusion of family as a random effect produced modest improvements in model fit for all nephron measurements

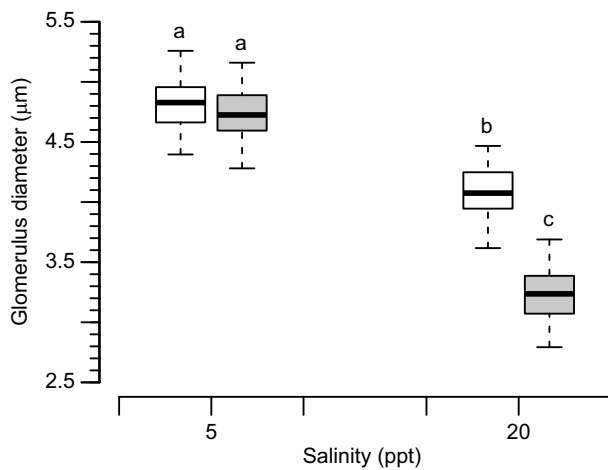


Fig. 1. Mean glomerulus diameter, conditioned on broad-sense genetic variation and random variation due to measurement error. $N=12$ per treatment. KAS(L) is plotted in white, MAR(H) in gray. Whiskers denote 95% PDIs; boxes indicate the quartile range of posterior estimates. Estimates that differ significantly are indicated by different lowercase letters. KAS, Kaskinen; L, low salinity; MAR, Mariager Fjord; H, high salinity; PDI, posterior density interval estimates.

(Table 2). This was restricted to variation in the intercept for most traits, with the exception of tubule cell height, which also exhibited family-specific variation in coefficients describing the effect of rearing salinity – this is also reflected in the broader density interval estimates observed in the 20 ppt environment (Fig. 2C). However, given the small numbers of families sampled, posterior density interval estimates for family-level variation ranged over an order of magnitude. Based on point estimates (i.e. the posterior mode), variance due to measurement error was generally greater than that associated with family, although relative to the scale of structures measured was not excessive – from 9% to 16% when expressed as standard deviation relative to the global mean. Similar levels of variation were observed among the various kidney sections.

Candidate gene expression

When reared at 5 ppt salinity, transcript abundance did not differ significantly between populations for any of the six candidate genes. Four of the candidate genes did exhibit population-specific trends in their transcriptional response to salinity (Fig. 3), although under a stringent threshold ($\alpha=0.05$), a population-by-treatment interaction was significant only for *AQP3A* expression (Table 3). Similar trends were seen for *ATP1A1* and *NKCC2* under a more liberal significance threshold ($\alpha=0.1$), and qualitatively for *CD2AP*. MAR(H) showed a 1.7-fold increase in *AQP3A* transcription in response to salinity ($P=0.024$) whereas KAS(L) showed no change (Fig. 3A). This was also reflected in a 2.4-fold difference in transcript abundance between populations reared at 20 ppt ($P=0.024$). Both *ATP1A1* and *CD2AP* appeared to be upregulated in MAR(H) individuals reared under high salinity (Fig. 3B,C), although neither observation was significant ($P=0.268$ and $P=0.250$, respectively). Nevertheless, within the 20 ppt treatment, *ATP1A1* transcript abundance was 1.4-fold higher in MAR(H) than KAS(L) (Table 3; $P=0.010$). In contrast, *NKCC2* expression was significantly reduced in KAS(L) individuals reared at high salinity (Fig. 3F; $P=0.022$) whereas no salinity effects were observed for MAR(H). Consequently, *NKCC2* transcript abundance was 1.5-fold greater in MAR(H) compared with KAS(L) at 20 ppt. No significant salinity effects or population differences were observed for *NEPH3* (Fig. 3D) or *NHE3B* (Fig. 3E).

Model fit was only marginally improved by incorporating family as a random term; for *NHE3B* the model accounting only for measurement error was preferred (Table 3). No candidate gene exhibited family-specific variation in their transcriptional response to salinity. Conversely, variance due to measurement error was substantially greater than that attributable to broad-sense genetic variation in expression.

DISCUSSION

The main objective of our study was to shed light on putative osmoregulatory mechanisms in the threespine stickleback, focusing on wild populations adapted to divergent conditions within a natural salinity gradient of the Baltic Sea (DeFaveri and Merilä, 2013, 2014). Specifically, we aimed to determine if there were structural and functional differences in the kidney between populations native to different salinity environments, and whether this variation was genetically based. Current results are insufficient for a formal test to distinguish selection from drift in the evolution of these populations; however, the observation of broad-sense genetic variation underlying traits essential to osmoregulatory function alludes to population-specific responses to selective pressures exerted by divergent salinity conditions. Although the Bay of Bothnia from which the low-salinity population originate is not fully freshwater, the low-salinity conditions are stable throughout the year (Håkansson et al., 1996). Moreover, the waters of this region are hypotonic (5 ppt salinity; $100\text{--}150\text{ mOsm kg}^{-1}$) relative to the blood plasma of sticklebacks (ca. 330 mOsm kg^{-1} ; De Ruiter and Wendelaar Bonga, 1985), a contrast that has produced osmotic stress in kidney-derived cell lines of the euryhaline spotted scat (Gui et al., 2016) and at the whole-organism level in other marine species, such as the green crab (Lovett et al., 2001, 2006). Likewise, this weak salinity has been proven to limit survival in other marine/estuarine fishes such as the estuary seahorse (*Hippocampus kuda*; Hilomen-Garcia et al., 2003), cobia (*Rachycentron canadum*; Resley et al., 2006) and weakfish (*Cynoscion regalis*; Lankford and Targett, 1994), all of which experienced significant mortality upon exposure to 5 ppt. Conversely, marine killifish (*Fundulus heteroclitus*) exposed to 5 ppt did not exhibit a loss of osmotic balance (Whitehead et al., 2011), suggesting that the degree to which 5 ppt represents a truly hypotonic stress likely varies among species. Nevertheless, when taken together with earlier results of contrasting tolerance in sticklebacks also reared under the same high and low salinities (DeFaveri and Merilä, 2014), there is evidence to suggest that these osmotic conditions indeed represent divergent selective pressures.

Our analyses also revealed a high degree of plasticity in the kidneys of individuals native to a high-salinity habitat, as most morphological features and some gene expression patterns showed significant responses (in the expected direction) to salinity treatment. However, this plasticity appears to be reduced in sticklebacks residing in a low-salinity environment, as most of the renal morphological and transcriptional traits examined did not show salinity-dependent responses. This suggests that pre-existing plasticity in the marine population has enabled the ancestral population to respond to abrupt salinity stress, but this ability has been lost in the locally adapted low-salinity population.

Kidney morphology

In line with earlier observations (Ogawa, 1968; Wendelaar Bonga, 1973), we also noted a high degree of plasticity in the glomeruli and nephron tubules from sticklebacks native to the high-salinity environment of the Kattegat. Specifically, when reared under low-

Table 2. Evaluation of mixed-effects models describing variation in nephron features as a function of population of origin (Pop), salinity exposure (Treat) and their interaction

Nephron feature	Fixed effects		Random effects		Variance estimates			
	Model term	P-value	Model	DIC	Variance	Post. mode	95% PDIs	
Glomerulus diameter	Pop	0.734	null	142.9	V_{meas}	0.143	0.101	0.186
	Treat	<0.001	V_G	140.8	$V_{\beta_0 \text{Fam}}$	0.073	0.006	0.699
	Pop×treat	<0.001	$V_G \times E$	140.9	V_{resid}	0.055	0.055	0.070
	Simple models							
	5 ppt: L vs H	0.706						
	20 ppt: L vs H	0.026						
	KAS(L): salinity	<0.001						
	MAR(H): salinity	<0.001						
Tubule diameter	Pop	0.150	null	1661.9	V_{meas}	0.179	0.161	0.201
	Treat	0.940	V_G	1661.3	V_{sect}	0.278	0.215	0.340
	Pop×treat	<0.001	$V_G \times E$	1661.3	$V_{\beta_0 \text{Fam}}$	0.069	0.015	0.721
	Simple models				V_{resid}	0.067	0.064	0.069
	5 ppt: L vs H	0.256						
	20 ppt: L vs H	0.286						
	KAS(L): salinity	0.984						
	MAR(H): salinity	<0.001						
Tubule lumen diameter	Pop	0.260	null	−6888.7	V_{meas}	0.059	0.052	0.064
	Treat	0.198	V_G	−6890.0	V_{sect}	0.056	0.043	0.070
	Pop×treat	<0.001	$V_G \times E$	−6888.7	$V_{\beta_0 \text{Fam}}$	0.038	0.010	0.310
	Simple models				V_{resid}	0.015	0.014	0.016
	5 ppt: L vs H	0.140						
	20 ppt: L vs H	0.560						
	KAS(L): salinity	0.062						
	MAR(H): salinity	<0.001						
Tubule cell height	Pop	0.376	null	−8947.5	V_{meas}	0.022	0.020	0.025
	Treat	0.776	V_G	−8947.7	V_{sect}	0.025	0.019	0.031
	Pop×treat	0.332	$V_G \times E$	−8948.1	$V_{\beta_0 \text{Fam}}$	0.013	0.003	0.120
					$V_{\beta_{\text{Treat}} \text{Fam}}$	0.019	0.006	0.242
					V_{resid}	0.011	0.010	0.011

For features with a significant population-by-treatment interaction, results of reduced models with contrasting population differences (L vs H) in each salinity, as well as salinity effects within each population, are also presented. All models account for measurement error as variance attributable to repeated measures of the same feature (V_{meas}), and for tubule measurements, variation among histological sections (V_{sect}). V_{resid} denotes residual/error variance. The significance of random model terms was evaluated in a forward step-wise process, first incorporating broad-sense genetic variation (V_G) as among-family variation in the model intercept ($V_{\beta_0|\text{Fam}}$), followed by inclusion of family-specific slopes ($V_{\beta_{\text{Treat}}|\text{Fam}}$) to capture broad-sense genotype-by-environment interactions ($V_G \times E$). Variance estimates are profiled from the parsimony model, indicated by the deviance information criterion (DIC) value in bold font; the posterior mode (Post. mode) and 95% posterior density interval estimates (95% PDIs) are presented.

salinity conditions, most renal morphological traits showed a significant increase in diameter. In fact, the tubules of the high-salinity population were even larger than those of the low-salinity population when both were reared in low salinity, reflecting their enhanced ability to respond to changes in environmental salinity, in this case by likely increasing filtration rate in low salinity to conserve filtrated electrolytes and re-absorb ions (Hickman and Trump, 1969). Accordingly, many studies have reported similar or better performance (e.g. hatching success, larval survival, growth) of marine/anadromous sticklebacks reared in low as compared with high salinity (McCairns and Bernatchez, 2010; McGuigan et al., 2011; DeFaveri and Merilä, 2014), suggesting that ancestral sticklebacks maintain the functional plasticity that likely permitted exploitation of novel freshwater habitats during initial colonization. Hence, our results indicate that part of this plasticity is likely achieved through modification of key renal structures.

To the best of our knowledge, no histological study to date has investigated the effects of high salinity transfer on the kidney of freshwater/low-salinity sticklebacks. In this regard, our study provides novel insights into the reduction of plasticity in derived populations: although there was a treatment effect on glomeruli diameter in KAS(L) fish reared under high salinity, the reduction in glomerulus size was significantly less pronounced than that observed from conspecifics of marine origin. This could suggest a

reduced osmoregulatory capacity in high-salinity conditions, as structural differences in the kidney are widely recognized to reflect functional differences in ion transport (Hickman and Trump, 1969; Lam and Leatherland, 1969; Wendelaar Bonga, 1976; Reimschuessel, 2001). Indeed, reducing glomerulus size is one means by which marine teleosts reduce plasma filtration to produce more concentrated urine (McDonald, 2007). Likewise, in contrast to marine fish which reduced overall tubule and tubule lumen diameter when reared under high salinity, no modifications were observed in the tubules of KAS(L) fish. As there is a direct relationship between tubule diameter and glomerular filtration rate (Wong and Woo, 2006), reduction in tubule size is associated with osmoregulation in hypertonic environments. Moreover, as water reabsorption from urine occurs in the tubules (McDonald, 2007), it stands to reason that a reduction in tubule diameter should prolong exposure to the villi responsible for absorption. Thus, failure to sufficiently reduce glomerular filtration rate and/or increase tubular water reabsorption may represent a disruption to normal homeostasis that could explain fitness reductions observed when freshwater/low-salinity sticklebacks are reared in high salinity, including reduced hatching rates (Gutz, 1970; Marchinko and Schluter, 2007), increased mortality (McCairns and Bernatchez, 2010; DeFaveri and Merilä, 2014) and smaller body size (DeFaveri and Merilä, 2014).

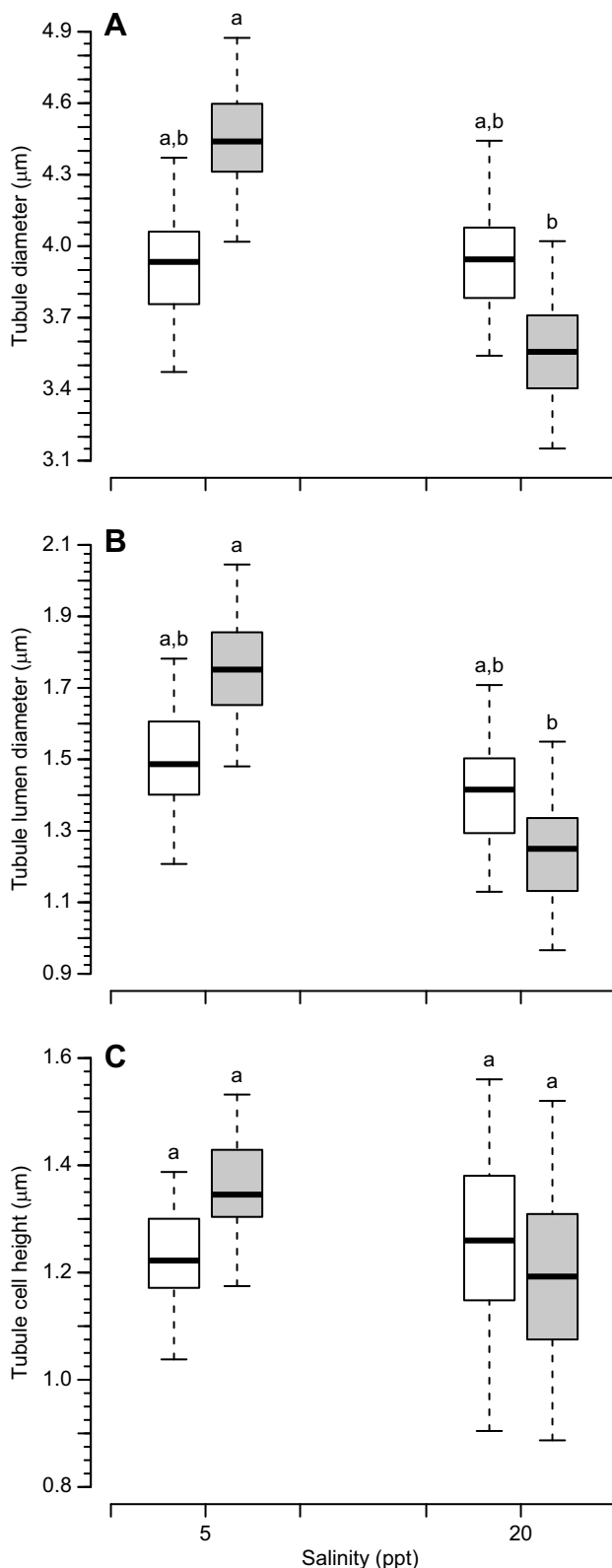


Fig. 2. Tubule characteristics, conditioned on broad-sense genetic variation, longitudinal variation within a given kidney and random variation due to measurement error ($N=12$ per treatment). KAS(L) is plotted in white, MAR(H) in gray. Whiskers denote 95% PDIs; boxes indicate the quartile range of posterior estimates. (A) Tubule diameter; (B) lumen diameter; (C) cell height. Estimates that differ significantly are indicated by different lowercase letters. KAS, Kaskinen; L, low salinity; MAR, Mariager Fjord; H, high salinity; PDI, posterior density interval estimates.

These contrasting patterns suggest that plasticity in renal development and/or capacity for remodeling has been significantly reduced (glomerulus size) – and to some extent perhaps even lost (tubule diameter) – in the population residing permanently in a low-salinity environment. Loss/reduction of plasticity would seem to be a common feature in sticklebacks following colonization of freshwater, with similar patterns observed at both the transcriptional and morphological level in separate systems (Wund et al., 2008; McCairns and Bernatchez, 2010), and speaks to the broader role of plasticity potentially facilitating the transition to permanent freshwater residency (West-Eberhard, 2005; Crispo, 2008). One explanation could be that osmoregulatory plasticity may be costly to maintain, but nevertheless adaptive for populations such as MAR(H) that reside in fluctuating environments characterized by daily salinity variations due to tidal cycles (salinity ranges from 17 to 24 ppt) and even more dramatic seasonal changes associated with spawning and egg rearing in freshwater. Adaptation to the relative osmotic stability of the low-salinity environment should select for more canalized and/or specialist genotypes (DeWitt et al., 1998; Kvitek and Sherlock, 2013; Murren et al., 2015). Conversely, residency in a less heterogeneous environment may simply reflect a relaxation of selection, which in turn might yield a loss of plasticity via random mutational processes (Masel et al., 2007; Maughan et al., 2007). In the absence of data on bioenergetic and/or fitness costs associated with plasticity (or the lack thereof), we cannot provide a definitive explanation for the contrasting patterns observed between marine and low-salinity populations. However, given the preponderance of observations involving the loss of plasticity across multiple phenotypes in the stickleback system, we would encourage their inclusion in future studies.

Gene expression

In general, variation in the expression patterns of most candidate genes studied here coincided with the structural changes discussed above. Although not significant, similar trends were observed in the high-salinity population, which was able to downregulate three genes (*AQP3A*, *ATP1A1* and *CD2AP*) in the low-salinity treatment, suggesting some degree of plasticity in ion-transporting mechanisms in this population. Such transcriptional modifications were not observed in the low-salinity population (with the exception of *NKCC2*). Similar patterns have been reported in other fishes from the Baltic Sea area. For example, microarray analysis of liver tissues in flounder from the North Sea (33 ppt) uncovered 2.3% of genes that were differentially expressed after long-term exposure to different salinity treatments (33 and 9 ppt); although a formal enrichment analysis was not conducted, it was reported that many genes were known to play a role in osmoregulation (Larsen et al., 2007). In contrast, only 0.5% (an amount that could be expected by chance alone) were differentially expressed in flounder originating from the low brackish conditions of the inner Baltic Sea (9 ppt). Subsequent validation of candidate gene expression (including *ATP1A1*) in kidney and gill tissues from the same samples also revealed patterns of reduced responsiveness of Baltic Sea fish to salinity challenge, relative to their truly marine conspecifics from the North Sea (Larsen et al., 2008). Assays of *ATP1A1* transcription in gill tissues of Atlantic cod also showed a concordant result: significant changes were observed in response to salinity treatment in the North Sea population but not in the Baltic Sea population (Larsen et al., 2012). In general, it appears that populations of fishes have become locally adapted to the low-salinity conditions of the Baltic Sea, and this is reflected in their inability to modify transcriptional responses to salinity changes.

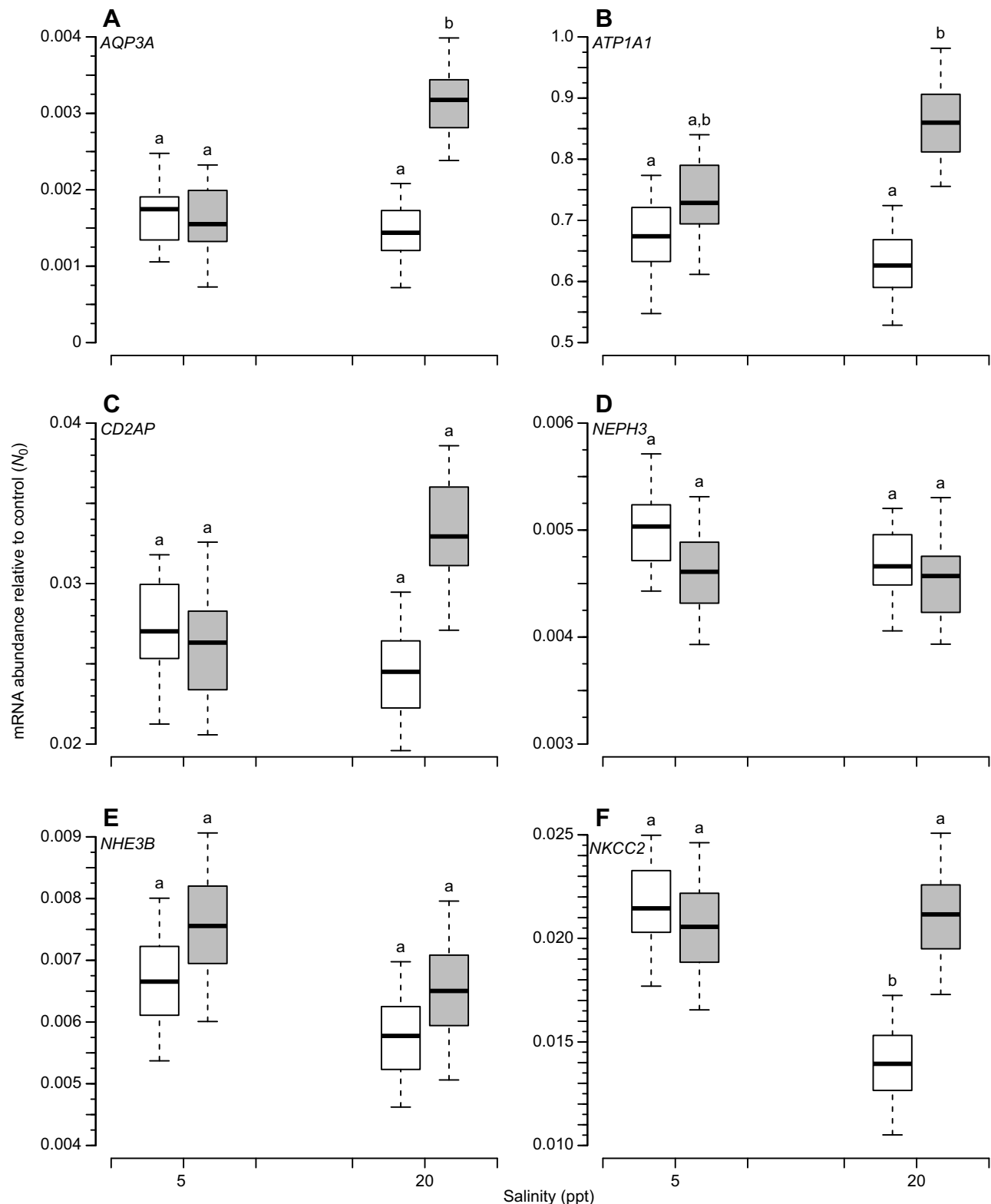


Fig. 3. Mean initial mRNA concentrations (N_0), conditioned on broad-sense genetic variation and random variation due to measurement error ($N=30-40$ per group). KAS(L) is plotted in white, MAR(H) in gray. Whiskers denote 95% PDIs; boxes indicate the quartile range of posterior estimates. Estimates that differ significantly are indicated by different lowercase letters. KAS, Kaskinen; L, low salinity; MAR, Mariager Fjord; H, high salinity; PDI, posterior density interval estimates.

Of the six genes tested here, expression of *ATP1A1* is perhaps the most studied in the context of osmoregulation – not just among sticklebacks (e.g. McCairns and Bernatchez, 2010; Judd, 2012; Taugbøl et al., 2014; Wang et al., 2014; Divino et al., 2016), but also

in many other euryhaline species, such as killifish (*F. heteroclitus*; Whitehead et al., 2012), Japanese eel (*Anguilla japonica*; Tang et al., 2012), Senegalese sole (*Solea senegalensis*; Armesto et al., 2014), Atlantic salmon (*Salmo salar*; Bystriansky and Schulte,

Table 3. Evaluation of mixed-effects models describing variation in initial mRNA concentration as a function of population of origin (Pop), salinity exposure (Treat) and their interaction

Candidate gene	Fixed effects		Random effects		Variance estimates			
	Model term	P-value	Model	DIC	Variance	Post. mode	95% PDIs	
<i>AQP3</i>	Pop	0.746	null	−4070.2	V_{meas}	5.2×10^{-6}	3.9×10^{-6}	6.7×10^{-6}
	Treat	0.542	V_G	−4070.4	V_{plate}	2.6×10^{-7}	2.0×10^{-7}	3.4×10^{-7}
	Pop×treat	0.020	$V_{G \times E}$	−3947.9	$V_{\beta_0 Fam}$	1.6×10^{-9}	9.6×10^{-17}	1.7×10^{-6}
	Simple models				V_{resid}	4.7×10^{-7}	4.2×10^{-7}	5.0×10^{-7}
	5 ppt: L vs H	0.542						
	20 ppt: L vs H	0.024						
	KAS(L): salinity	0.280						
<i>ATP1A1</i>	MAR(H): salinity	0.040						
	Pop	0.514	null	−1064.1	V_{meas}	1.3×10^{-1}	1.0×10^{-1}	1.7×10^{-1}
	Treat	0.552	V_G	−1064.2	V_{plate}	2.1×10^{-2}	1.7×10^{-2}	2.7×10^{-2}
	Pop×treat	0.099	$V_{G \times E}$	−1036.4	$V_{\beta_0 Fam}$	7.3×10^{-8}	1.3×10^{-16}	1.4×10^{-3}
	Simple models				V_{resid}	1.9×10^{-2}	1.7×10^{-2}	2.0×10^{-2}
	5 ppt: L vs H	0.570						
	20 ppt: L vs H	0.010						
<i>CD2AP</i>	KAS(L): salinity	0.388						
	MAR(H): salinity	0.268						
	Pop	0.874	null	−7250.3	V_{meas}	3.7×10^{-4}	2.7×10^{-4}	4.6×10^{-4}
	Treat	0.570	V_G	−7255.0	V_{plate}	1.3×10^{-5}	6.0×10^{-6}	3.0×10^{-5}
<i>KIRREL3L</i> (<i>NEPH3</i>)	Pop×treat	0.202	$V_{G \times E}$	−7132.7	$V_{\beta_0 Fam}$	1.9×10^{-9}	7.4×10^{-17}	9.3×10^{-6}
					V_{resid}	1.4×10^{-4}	1.2×10^{-4}	1.5×10^{-4}
	Pop	0.478	null	−3627.2	V_{meas}	5.2×10^{-6}	3.8×10^{-6}	6.4×10^{-6}
	Treat	0.490	V_G	−3627.5	V_{plate}	7.4×10^{-7}	5.8×10^{-7}	9.0×10^{-7}
<i>SLC9A3.2</i> (<i>NHE3B</i>)	Pop×treat	0.678	$V_{G \times E}$	−3414.3	$V_{\beta_0 Fam}$	6.9×10^{-11}	1.2×10^{-16}	1.4×10^{-7}
					V_{resid}	5.9×10^{-7}	5.5×10^{-7}	6.7×10^{-7}
	Pop	0.464	null	−9971.8	V_{meas}	2.0×10^{-5}	1.5×10^{-5}	2.6×10^{-5}
	Treat	0.434	V_G	−9971.7	V_{plate}	1.0×10^{-5}	7.7×10^{-6}	1.2×10^{-5}
<i>SLC12A1</i> (<i>NKCC2</i>)	Pop×treat	0.912	$V_{G \times E}$	−9892.6	$V_{\beta_0 Fam}$	2.6×10^{-10}	8.2×10^{-17}	7.1×10^{-7}
					V_{resid}	5.1×10^{-6}	4.6×10^{-6}	5.7×10^{-6}
	Pop	0.816	null	−7421.4	V_{meas}	1.5×10^{-4}	1.1×10^{-4}	2.0×10^{-4}
	Treat	0.012	V_G	−7421.6	V_{plate}	2.6×10^{-5}	1.4×10^{-5}	3.6×10^{-5}
	Pop×treat	0.065	$V_{G \times E}$	−7298.5	$V_{\beta_0 Fam}$	7.0×10^{-10}	1.1×10^{-16}	1.2×10^{-6}
	Simple models				V_{resid}	1.2×10^{-4}	1.1×10^{-4}	1.3×10^{-4}
	5 ppt: L vs H	0.792						
	20 ppt: L vs H	0.014						
	KAS(L): salinity	0.022						
	MAR(H): salinity	0.832						

Layout and estimates are the same as in Table 2; note that plate effects (V_{plate}) are also included in models and variance estimates.

2011) and sockeye salmon (*Oncorhynchus nerka*; Shrimpton et al., 2005); see Havird et al. (2013) for a meta-analysis. A common finding among these studies is the upregulation in seawater/high salinity (20–33 ppt) as compared with freshwater/low salinity (but see McCairns and Bernatchez, 2010), a trend that was also seen in our study – although only in the population native to high salinity. However, in sticklebacks, the population-specificity of *ATP1A1* expression is less consistent among studies that have used gill tissue rather than kidney. For example, some report that plasticity has been retained in freshwater populations, which are able to upregulate Na^+/K^+ -ATPases in seawater (compared with freshwater) at a similar level as native seawater populations (e.g. Judd, 2012; Taugbøl et al., 2014). Interestingly, a recent study of wild-caught sticklebacks did note changes in *ATP1A1* expression in the kidneys of freshwater sticklebacks acclimated to different salinities (0, 11 and 33 ppt), although these expression patterns were dissimilar to the current study; that is, whereas we observed no significant effect of salinity on *ATP1A1* transcription in fish originating from a low-salinity environment (a slight trend for mean expression to decrease was observed, Fig. 3B), Wang et al. (2014) demonstrated a monotonic decrease in expression of freshwater fish when exposed to increasing salinity. Nevertheless, there are also similarities between studies, including both elevated levels of *ATP1A1* mRNA

in saltwater fish exposed to a hypertonic environment, relative to their low-salinity conspecifics, as well as generally greater variability in levels of expression for fish of marine origin.

In addition to ion transport, osmotic balance is also maintained through regulation of cell water volume, a function performed by aquaporins. Not surprisingly, the most significant transcriptional change we observed was in *AQP3A*, for which mRNA levels were 2.4-fold higher in the high-salinity treatment. Similar to *ATP1A1* and *CD2AP*, this upregulation in the high-salinity treatment was only observed in the high-salinity population. Interestingly, this expression pattern is opposite to what is generally observed: with the exception of a small sample of tilapia (*Oreochromis mossambicus*; Watanabe et al., 2005), most euryhaline fish tend to upregulate *AQP3* isoforms in freshwater (see Cutler et al., 2007 for a review of *AQP3* in teleosts). Such expression differences could reflect localized functional differences in various tubule segments, e.g. the proximal versus distal renal tubules, which have different roles in fluid secretion and water permeability (Beyenbach, 2004). The role of *AQP3* in the kidney of teleosts has been suggested to reabsorb water/fluid from the distal tubular segment (Cutler et al., 2007); importantly, however, the distal nephron appears to be absent in sticklebacks (Elger et al., 2000). Therefore, further study may be needed to clarify the precise role of *AQP3* in teleost kidneys. Using

RNA sequencing, Wang et al. (2014) identified *AQP4*, another member of the aquaporin family, as a salt-responsive gene in the kidneys of sticklebacks, although significant differences were only observed in seawater when comparing marine and freshwater sticklebacks acclimated to seawater (33 ppt). Notably, no significant differences were observed in the expression levels among freshwater sticklebacks acclimated to different salinities (0, 11 and 33 ppt; Wang et al., 2014), a trend we also observed in *AQP3A* (Fig. 3A). Hence, this lack of transcriptional response to salinity in freshwater sticklebacks aligns with our results for aquaporins. Unfortunately, Wang et al. (2014) were not able to include data from marine sticklebacks acclimated to salinities other than their native seawater, so the plasticity of aquaporin expression in this population of marine sticklebacks cannot be assessed.

Linking gene expression and the morphological remodeling of kidney tissue

Structural remodeling of the kidney has been associated with changes in levels of mRNA expression in a number of animals, including changes in tubule size in *Xenopus* (Cerqueira et al., 2014) and glomerulus structure in mice (Hayashi et al., 2014). Conversely, mechanically induced remodeling independent of genetic causes may also induce changes in transcription (Mammoto et al., 2013), an observation that further emphasizes caveats against conflating correlation and causation. Yet such patterns can nevertheless be informative; and although organ size precluded the simultaneous measurement of mRNA abundance and kidney structure in the same individual, our observations are suggestive of such a correspondence.

With the exception of *NKCC2*, we observed no significant transcriptional changes in the low-salinity population when reared under high salinity, a pattern also coincident with the observation of far less plasticity in the kidney structure of this group. However, the high-salinity population demonstrated a marked response to salinity both in renal structures and transcript abundance. Both observations suggest correlation and/or causation between cellular remodeling and active transport mechanisms during osmotic acclimation. Indeed, similar links have been shown in the gill epithelium of killifish, which can transition from a seawater to freshwater phenotype in less than one day, with corresponding changes in ion-transporting functions (Whitehead et al., 2012, 2013). Although less well studied in fishes, structural changes in the kidney can also implicate activity-associated modifications in ion transport and metabolism (Hickman and Trump, 1969; Beyenbach, 2004). For example, changes in the volume of multiple cell types, which affect water flow and/or solute concentrations, can be achieved by activating various channels and transporters, including sodium and potassium transporters such as *ATP1A1* and *NKCC2* (Okada, 2004). Likewise, epithelial remodeling in the kidney of puffer fish (*Tetraodon biocellatus*) acclimated to different salinities revealed concordance between claudin tight junction protein mRNA abundance and structural changes observed in nephron tubules and tight junctions (Duffy et al., 2011). It is therefore possible that similar links are also important in stickleback kidneys. For example, given that aquaporins are associated with both tubule cell volume regulation and water reabsorption (Day et al., 2014), the pattern of increased expression in high salinity (Fig. 3A) could potentially be correlated with changes in tubule structure found in our study (Fig. 2). Hence, the effects of varying and/or silencing aquaporin expression on kidney structure and function poses one potential mechanistic link worth future study. Again, we must reiterate that separating cause and correlation requires work well beyond that undertaken here, and a clear link between cellular remodeling and

expression in sticklebacks remains to be confirmed. Although as tools for targeted mutagenesis and RNA silencing become more prevalent, such a link may prove attainable.

Considerations, perspectives and conclusions

There are several points worth considering when interpreting the results presented here. Firstly, as we only used F1-generation individuals, it is not possible to rule out variation attributable to maternal, complex developmental or epigenetic effects. Further studies with multi-generation controlled breeding might help to more carefully differentiate between these sources of variation. Secondly, there are distinct roles in active secretion and reabsorption of various ions and water in different parts of the kidney tubules and collecting ducts. The distal segment is absent in sticklebacks, and we were not able to distinguish different proximal segments and collecting ducts (using standard antibodies, e.g. Calbindin, Pax2, PNA, HRP, TH and Na⁺/K⁺-ATPase; S.K. and M.M.H., unpublished data). Therefore, the inferences made from kidney tubule morphology are an approximation over the entire tubule, rather than precise morphological analysis of different tubular segments. Thirdly, gene expression was measured by quantification of mRNA abundance. Although there can be a generally high correspondence between mRNA and protein expression (Greenbaum et al., 2003), this has yet to be verified in this study. It should be noted, however, that in sticklebacks *ATP1A1* enzyme activity has been demonstrated to increase in response to salinity challenge (Divino et al., 2016). Nevertheless, until mRNA concentration can be demonstrated to correspond to either the abundance and/or the functional activity of the protein in all candidate genes, our results should be interpreted with caution. Finally, rather than several candidate genes, complex genetic networks are involved in gene expression-driven adaptive divergence (Pavey et al., 2010; Whitehead et al., 2013). Therefore, further studies investigating the whole transcriptome may help to build a more comprehensive understanding of the complex physiological processes involved with osmoregulatory plasticity and adaptation.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: J.D., J.M., R.J.S.M.; Formal analysis: M.M.H., R.J.S.M.; Investigation: M.M.H., S.K., S.N.D., R.J.S.M.; Resources: J.D., S.K., S.L., J.M.; Data curation: R.J.S.M.; Writing - original draft: M.M.H., J.D., R.J.S.M.; Writing - review and editing: M.M.H., J.D., S.K., S.N.D., S.L., J.M., R.J.S.M.; Supervision: R.J.S.M.; Project administration: R.J.S.M.; Funding acquisition: R.J.S.M., J.M.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.146027.supplemental>

References

- Abramoff, M. D., Magalhães, P. J. and Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics Int.* **11**, 36–42.
- Armesto, P., Campinho, M. A., Rodríguez-Rúa, A., Cousin, X., Power, D. M., Manchado, M. and Infante, C. (2014). Molecular characterization and

- transcriptional regulation of the Na⁺/K⁺ ATPase α subunit isoforms during development and salinity challenge in a teleost fish, the Senegalese sole (*Solea senegalensis*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **175**, 23–38.
- Bell, M. A. and Foster, S. A.** (1994). Introduction to the evolutionary biology of stickleback. In *The Evolutionary Biology of the Threespine Stickleback* (ed. M. A. Bell and S. A. Foster), pp. 1–27. Oxford: Oxford University Press.
- Beyenbach, K. W.** (2004). Kidneys sans glomeruli. *Am. J. Physiol. Renal Physiol.* **286**, F811–F827.
- Bystriansky, J. S. and Schulte, P. M.** (2011). Changes in gill H⁺-ATPase and Na⁺/K⁺-ATPase expression and activity during freshwater acclimation of Atlantic salmon (*Salmo salar*). *J. Exp. Biol.* **214**, 2435–2442.
- Cerqueira, D. M., Tran, U., Romaker, D., Abreu, J. G. and Wessely, O.** (2014). Sterol carrier protein 2 regulates proximal tubule size in the *Xenopus* pronephric kidney by modulating lipid rafts. *Dev. Biol.* **394**, 54–64.
- Chang, W.-J., Wang, Y.-F., Hu, H.-J., Wang, J.-H., Lee, T.-H. and Hwang, P.-P.** (2013). Compensatory regulation of Na⁺ absorption by Na⁺/H⁺ exchanger and Na⁺-Cl⁻ cotransporter in zebrafish (*Danio rerio*). *Front. Zool.* **10**, 46.
- Čikoš, Š., Bukovská, A. and Koppel, J.** (2007). Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis. *BMC Mol. Biol.* **8**, 113.
- Crispo, E.** (2008). Modifying effects of phenotypic plasticity on interactions among natural selection, adaptation and gene flow. *J. Evol. Biol.* **21**, 1460–1469.
- Cutler, C. P. and Cramb, G.** (2001). Molecular physiology of osmoregulation in eels and other teleosts: the role of transporter isoforms and gene duplication. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **130**, 551–564.
- Cutler, C. P., Martinez, A.-S. and Cramb, G.** (2007). The role of aquaporin 3 in teleost fish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **148**, 82–91.
- Dalziel, A. C., Rogers, S. M. and Schulte, P. M.** (2009). Linking genotypes to phenotypes and fitness: how mechanistic biology can inform molecular ecology. *Mol. Ecol.* **18**, 4997–5017.
- Day, R. E., Kitchen, P., Owen, D. S., Bland, C., Marshall, L., Conner, A. C., Bill, R. M. and Conner, M. T.** (2014). Human aquaporins: regulators of transcellular water flow. *BBA Gen. Subjects* **1840**, 1492–1506.
- De Ruiter, A. J. H. and Wendelaar Bonga, S. E.** (1985). Consequences of nestbuilding behaviour for osmoregulation in male three-spined sticklebacks. *Behaviour* **93**, 8–20.
- DeFaveri, J. and Merilä, J.** (2013). Evidence for adaptive phenotypic differentiation in Baltic Sea sticklebacks. *J. Evol. Biol.* **26**, 1700–1715.
- DeFaveri, J. and Merilä, J.** (2014). Local adaptation to salinity in the three-spined stickleback? *J. Evol. Biol.* **27**, 290–302.
- DeFaveri, J., Shikano, T., Shimada, Y., Goto, A. and Merilä, J.** (2011). Global analysis of genes involved in freshwater adaptation in threespine sticklebacks (*Gasterosteus aculeatus*). *Evolution* **65**, 1800–1807.
- DeFaveri, J., Jonsson, P. R. and Merilä, J.** (2013). Heterogeneous genomic differentiation in marine threespine sticklebacks: adaptation along an environmental gradient. *Evolution* **67**, 2530–2546.
- DeWitt, T. J., Sih, A. and Wilson, D. S.** (1998). Costs and limits of phenotypic plasticity. *Trends Ecol. Evol.* **13**, 77–81.
- Divino, J. N., Monette, M. Y., McCormick, S. D., Yancey, P. H., Flannery, K. G., Bell, M. A., Rollins, J. L., von Hippel, F. A. and Schultz, E. T.** (2016). Osmoregulatory physiology and rapid evolution of salinity tolerance in threespine stickleback recently introduced to fresh water. *Evol. Ecol. Res.* **17**, 179–201.
- Duffy, N. M., Bui, P., Bagherie-Lachidan, M. and Kelly, S. P.** (2011). Epithelial remodeling and claudin mRNA abundance in the gill and kidney of puffer fish (*Tetraodon lineatus*) acclimated to altered environmental ion levels. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **181**, 219–238.
- Eaton, D. C.** (2012). Frontiers in renal and epithelial physiology—grand challenges. *Front. Physiol.* **3**, 2.
- Eddy, F. B. and Handy, R. D.** (2012). *Ecological and Environmental Physiology of Fishes*. Oxford, UK: Oxford University Press.
- Elger, M., Hentschel, H., Dawson, M. and Renfro, J. L.** (2000). Urinary Tract. In *The Laboratory Fish* (ed. G. K. Ostrander), pp. 385–413. London: Academic Press.
- Engelund, M. B. and Madsen, S.** (2011). The role of aquaporins in the kidney of euryhaline teleosts. *Front. Physiol.* **2**, 51.
- Evans, D. H.** (2008). Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R704–R713.
- Evans, D. H., Piermarini, P. M. and Potts, W. T. W.** (1999). Ionic transport in the fish gill epithelium. *J. Exp. Zool.* **283**, 641–652.
- Evans, D. H., Piermarini, P. M. and Choe, K. P.** (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97–177.
- Finn, R. N. and Cerdà, J.** (2011). Aquaporin evolution in fishes. *Front. Physiol.* **2**, 44.
- Fischer, A. H., Jacobson, K. A., Rose, J. Zeller, R.** (2008). Hematoxylin and eosin staining of tissue and cell sections. *Cold Spring Harb. Protoc.* 2008, 3, pdb. prot4986.
- Gibson, G.** (2005). The synthesis and evolution of a supermodel. *Science* **307**, 1890–1891.
- Greenbaum, D., Colangelo, C., Williams, K. and Gerstein, M.** (2003). Comparing protein abundance and mRNA expression levels on a genomic scale. *Genome Biol.* **4**, 117.
- Grosell, M.** (2006). Intestinal anion exchange in marine fish osmoregulation. *J. Exp. Biol.* **209**, 2813–2827.
- Gui, L., Zhang, P., Liang, X., Su, M., Wu, D. and Zhang, J.** (2016). Adaptive responses to osmotic stress in kidney-derived cell lines from *Scatophagus argus*, a euryhaline fish. *Gene* **583**, 134–140.
- Guo, B., DeFaveri, J., Sotelo, G., Nair, A. and Merilä, J.** (2015). Population genomic evidence for adaptive differentiation in Baltic Sea three-spined sticklebacks. *BMC Biol.* **13**, 19.
- Gutz, M.** (1970). Experimental studies on adaptation to salinity shown by the different races of the three-spined stickleback (*Gasterosteus aculeatus* L.). *Internat. Rev. Gesamten Hydrobiol. Hydrogr.* **55**, 845–894.
- Hadfield, J. D.** (2010). MCMC methods for multi-response generalized linear mixed models: the MCMCglmm R package. *J. Stat. Softw.* **33**, 1–22.
- Håkansson, B., Alenius, P. and Brydsten, L.** (1996). Physical environment in the Gulf of Bothnia. *Ambio Special Report* no. 8, 5–12. <http://www.jstor.org/stable/25094544>
- Havird, J. C., Henry, R. P. and Wilson, A. E.** (2013). Altered expression of Na⁺/K⁺–ATPase and other osmoregulatory genes in the gills of euryhaline animals in response to salinity transfer: a meta-analysis of 59 quantitative PCR studies over 10 years. *Comp. Biochem. Physiol. D Genomics Proteomics* **8**, 131–140.
- Hayashi, K., Sasamura, H., Nakamura, M., Azegami, T., Oguchi, H., Sakamaki, Y. and Itoh, H.** (2014). KLF4-dependent epigenetic remodeling modulates podocyte phenotypes and attenuates proteinuria. *J. Clin. Invest.* **124**, 2523–2537.
- Hentschel, H., Elger, M., Dawson, M. and Renfro, J. L.** (2000). Urinary Tract. In *The Laboratory Fish* (ed. G. K. Ostrander), pp. 181–187. London: Academic Press.
- Hentschel, D. M., Mengel, M., Boehme, L., Liebsch, F., Albertin, C., Bonventre, J. V., Haller, H. and Schiffer, M.** (2007). Rapid screening of glomerular slit diaphragm integrity in larval zebrafish. *Am. J. Physiol. Renal Physiol.* **293**, F1746–F1750.
- Hickman, C. P. and Trump, B. F.** (1969). The kidney. In *Fish Physiology. Volume I. Excretion, Ion Regulation and Metabolism* (ed. W. S. Hoar and D. J. Randall), pp. 91–239. New York, NY: Academic Press.
- Hilomen-Garcia, G. V., Delos Reyes, R. and Garcia, C. M. H.** (2003). Tolerance of seahorse *Hippocampus kuda* (Bleeker) juveniles to various salinities. *J. Appl. Ichthyol.* **19**, 94–98.
- Hwang, P.-P., Lee, T.-H. and Lin, L.-Y.** (2011). Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **301**, R28–R47.
- Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J., Swofford, R., Pirun, M., Zody, M. C. and White, S.** (2012). The genomic basis of adaptive evolution in threespine sticklebacks. *Nature* **484**, 55–61.
- Judd, S.** (2012). Na⁺/K⁺-ATPase isoform regulation in three-spine stickleback (*Gasterosteus aculeatus*) during salinity acclimation. MSc thesis, College of Science and Health, DePaul University, Chicago, IL.
- Kato, A., Muro, T., Kimura, Y., Li, S., Islam, Z., Ogoshi, M., Doi, H. and Hirose, S.** (2011). Differential expression of Na⁺-Cl⁻ cotransporter and Na⁺-K⁺-Cl⁻ cotransporter 2 in the distal nephrons of euryhaline and seawater pufferfishes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, R284–R297.
- Kvitik, D. J. and Sherlock, G.** (2013). Whole genome, whole population sequencing reveals that loss of signaling networks is the major adaptive strategy in a constant environment. *PLoS Genet.* **9**, e1003972.
- Lahlou, B.** (1983). Intestinal transport and osmoregulation in fishes. In *Intestinal Transport* (ed. M. Gilles-Baillien and R. Gilles), pp. 341–353. Berlin: Springer.
- Lam, T. J. and Leatherland, J. F.** (1969). Effects of prolactin on the glomerulus of the marine threespine stickleback, *Gasterosteus aculeatus* L., form trachurus, after transfer from seawater to fresh water, during the late autumn and early winter. *Can. J. Zool.* **47**, 245–250.
- Lankford, T. E. and Targett, T. E.** (1994). Suitability of estuarine nursery zones for juvenile weakfish (*Cynoscion regalis*): effects of temperature and salinity on feeding, growth and survival. *Mar. Biol.* **119**, 611–620.
- Larsen, P. F., Nielsen, E. E., Williams, T. D., Hemmer-Hansen, J., Chipman, J. K., Kruhøffer, M., Gronkjaer, P., George, S. G., Dyrskjot, L. and Loeschcke, V.** (2007). Adaptive differences in gene expression in European flounder (*Platichthys flesus*). *Mol. Ecol.* **16**, 4674–4683.
- Larsen, P. F., Nielsen, E. E., Williams, T. D. and Loeschcke, V.** (2008). Intraspecific variation in expression of candidate genes for osmoregulation, heme biosynthesis and stress resistance suggests local adaptation in European flounder (*Platichthys flesus*). *Heredity* **101**, 247–259.
- Larsen, P. F., Nielsen, E. E., Meier, K., Olsvik, P. A., Hansen, M. M. and Loeschcke, V.** (2012). Differences in salinity tolerance and gene expression between two populations of Atlantic cod (*Gadus morhua*) in response to salinity stress. *Biochem. Genet.* **50**, 454–466.
- Lehtonen, S., Ora, A., Olkkonen, V. M., Geng, L., Zerial, M., Somlo, S. and Lehtonen, E.** (2000). In vivo interaction of the adapter protein CD2-associated protein with the type 2 polycystic kidney disease protein, polycystin-2. *J. Biol. Chem.* **275**, 32888–32893.

- Li, C., Ruotsalainen, V., Tryggvason, K., Shaw, A. S. and Miner, J. H. (2000). CD2AP is expressed with nephrin in developing podocytes and is found widely in mature kidney and elsewhere. *Am. J. Physiol. Renal Physiol.* **279**, F785–F792.
- Lovett, D. L., Verzi, M. P., Clifford, P. D. and Borst, D. W. (2001). Hemolymph levels of methyl farnesoate increase in response to osmotic stress in the green crab, *Carcinus maenas*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **128**, 299–306.
- Lovett, D. L., Tanner, C. A., Glomski, K., Ricart, T. M. and Borst, D. W. (2006). The effect of seawater composition and osmolality on hemolymph levels of methyl farnesoate in the green crab *Carcinus maenas*. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **143**, 67–77.
- Lu, W., Mayolle, A., Cui, G., Luo, L. and Balment, R. J. (2011). Molecular characterization and expression of α -globin and β -globin genes in the euryhaline flounder (*Platichthys flesus*). *Evid. Based Complement. Alternat. Med.* **2011**, 965153.
- Mammoto, T., Mammoto, A. and Ingber, D. E. (2013). Mechanobiology and developmental control. *Annu. Rev. Cell Dev. Biol.* **29**, 27–61.
- Marchinko, K. B. and Schluter, D. (2007). Parallel evolution by correlated response: lateral plate reduction in threespine stickleback. *Evolution* **61**, 1084–1090.
- Masel, J., King, O. D. and Maughan, H. (2007). The loss of adaptive plasticity during long periods of environmental stasis. *Am. Nat.* **169**, 38–46.
- Maughan, H., Masel, J., Birky, C. W. and Nicholson, W. L. (2007). The roles of mutation accumulation and selection in loss of sporulation in experimental populations of *Bacillus subtilis*. *Genetics* **177**, 937–948.
- McCairns, R. J. S. and Bernatchez, L. (2010). Adaptive divergence between freshwater and marine sticklebacks: insights into the role of phenotypic plasticity from an integrated analysis of candidate gene expression. *Evolution* **64**, 1029–1047.
- McDonald, M. D. (2007). The renal contribution to salt and water balance. In *Fish Osmoregulation* (ed. B. Baldisserotto, J. M. Mancera Romero and B. G. Kapoor), pp. 322–345. Boca Raton, USA: CRC Press.
- McGuigan, K., Nishimura, N., Currey, M., Hurwit, D. and Cresko, W. A. (2011). Cryptic genetic variation and body size evolution in threespine stickleback. *Evolution* **65**, 1203–1211.
- Murren, C. J., Auld, J. R., Callahan, H., Ghalambor, C. K., Handelsman, C. A., Heskell, M. A., Kingsolver, J. G., Maclean, H. J., Masel, J. and Maughan, H. (2015). Constraints on the evolution of phenotypic plasticity: limits and costs of phenotype and plasticity. *Heredity* **115**, 293–301.
- Nishimura, S., Yoshida, T., Sugiura, H., Shiota, S., Tsuchiya, K. and Nihei, H. (2004). CD2AP expression in a renal ischemia/reperfusion injury model and analysis of its related genes using overexpression and RNA interference technique. *Am. J. Nephrol.* **25**, 373–381.
- Ogawa, M. (1968). Seasonal difference of glomerular change of the marine form of the stickleback, *Gasterosteus aculeatus* L. after transferred into fresh water. *Sci. Rep. Saitama Univ. B* **5**, 117–123.
- Okada, Y. (2004). Ion channels and transporters involved in cell volume regulation and sensor mechanisms. *Cell Biochem. Biophys.* **41**, 233–258.
- Olenin, S. and Leppäkoski, E. (1999). Non-native animals in the Baltic Sea: alteration of benthic habitats in coastal inlets and lagoons. *Hydrobiologia* **393**, 232–243.
- Pavey, S. A., Collin, H., Nosil, P. and Rogers, S. M. (2010). The role of gene expression in ecological speciation. *Ann. N. Y. Acad. Sci.* **1206**, 110–129.
- Perry, S. F. and Fryer, J. N. (1997). Proton pumps in the fish gill and kidney. *Fish Physiol. Biochem.* **17**, 363–369.
- Perry, S. F., Shahsavari, A., Georgalis, T., Bayaa, M., Furimsky, M. and Thomas, S. L. Y. (2003). Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: their role in ionic and acid–base regulation. *J. Exp. Zool. Part A Comp. Exp. Biol.* **300A**, 53–62.
- Pough, F. H. (1989). Organismal performance and Darwinian fitness: approaches and interpretations. *Physiol. Zool.* **199**, 236.
- Reimschuessel, R. (2001). A fish model of renal regeneration and development. *ILAR J.* **42**, 285–291.
- Resley, M. J., Webb, K. A. and Holt, G. J. (2006). Growth and survival of juvenile coho, *Rachycentron canadum*, at different salinities in a recirculating aquaculture system. *Aquaculture* **253**, 398–407.
- Ristola, M. and Lehtonen, S. (2014). Functions of the podocyte proteins nephrin and Neph3 and the transcriptional regulation of their genes. *Clin. Sci.* **126**, 315–328.
- Schulte, P. M. (2001). Environmental adaptations as windows on molecular evolution. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **128**, 597–611.
- Shih, N.-Y., Li, J., Karpitskii, V., Nguyen, A., Dustin, M. L., Kanagawa, O., Miner, J. H. and Shaw, A. S. (1999). Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* **286**, 312–315.
- Shimada, Y., Shikano, T. and Merilä, J. (2011). A high incidence of selection on physiologically important genes in the three-spined stickleback, *Gasterosteus aculeatus*. *Mol. Biol. Evol.* **28**, 181–193.
- Shrimpton, J. M., Patterson, D. A., Richards, J. G., Cooke, S. J., Schulte, P. M., Hinch, S. G. and Farrell, A. P. (2005). Ionoregulatory changes in different populations of maturing sockeye salmon *Oncorhynchus nerka* during ocean and river migration. *J. Exp. Biol.* **208**, 4069–4078.
- Tang, C.-H., Lai, D.-Y. and Lee, T.-H. (2012). Effects of salinity acclimation on Na⁺/K⁺-ATPase responses and FXD11 expression in the gills and kidneys of the Japanese eel (*Anguilla japonica*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **163**, 302–310.
- Taugbøl, A., Arntsen, T., Østbye, K. and Vøllestad, L. A. (2014). Small changes in gene expression of targeted osmoregulatory genes when exposing marine and freshwater threespine stickleback (*Gasterosteus aculeatus*) to abrupt salinity transfers. *PLoS ONE* **9**, e106894.
- Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M. and Rozen, S. G. (2012). Primer3 – new capabilities and interfaces. *Nucleic Acids Res.* **40**, e115.
- Van Kleunen, M. and Fischer, M. (2005). Constraints on the evolution of adaptive phenotypic plasticity in plants. *New Phytol.* **166**, 49–60.
- Van Tienderen, P. H. (1997). Generalists, specialists, and the evolution of phenotypic plasticity in sympatric populations of distinct species. *Evolution* **51**, 1372–1380.
- Wang, H., Lehtonen, S., Chen, Y.-C., Heikkilä, E., Panula, P. and Holthöfer, H. (2012). Neph3 associates with regulation of glomerular and neural development in zebrafish. *Differentiation* **83**, 38–46.
- Wang, G., Yang, E., Smith, K. J., Zeng, Y., Ji, G., Connon, R., Fangue, N. A. and Cai, J. J. (2014). Gene expression responses of threespine stickleback to salinity: implications for salt-sensitive hypertension. *Front. Genet.* **5**, 312.
- Watanabe, S., Kaneko, T. and Aida, K. (2005). Aquaporin-3 expressed in the basolateral membrane of gill chloride cells in Mozambique tilapia *Oreochromis mossambicus* adapted to freshwater and seawater. *J. Exp. Biol.* **208**, 2673–2682.
- Watt, W. B. (1985). Bioenergetics and evolutionary genetics: opportunities for new synthesis. *Am. Nat.* **125**, 118–143.
- Wendelaar Bonga, S. E. (1973). Morphometrical analysis with the light and electron microscope of the kidney of the anadromous three-spined stickleback *Gasterosteus aculeatus*, form *trachurus*, from fresh water and from sea water. *Z. Zellforsch. Mikrosk. Anat.* **137**, 563–588.
- Wendelaar Bonga, S. E. (1976). The effect of prolactin on kidney structure of the euryhaline teleost *Gasterosteus aculeatus* during adaptation to fresh water. *Cell Tissue Res.* **166**, 319–338.
- West-Eberhard, M. J. (2005). Developmental plasticity and the origin of species differences. *Proc. Natl. Acad. Sci. USA* **102**, 6543–6549.
- Whitehead, A., Roach, J. L., Zhang, S. J. and Galvez, F. (2011). Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient. *Proc. Natl. Acad. Sci. USA* **108**, 6193–6198.
- Whitehead, A., Roach, J. L., Zhang, S. and Galvez, F. (2012). Salinity- and population-dependent genome regulatory response during osmotic acclimation in the killifish (*Fundulus heteroclitus*) gill. *J. Exp. Biol.* **215**, 1293–1305.
- Whitehead, A., Zhang, S., Roach, J. L. and Galvez, F. (2013). Common functional targets of adaptive micro- and macro-evolutionary divergence in killifish. *Mol. Ecol.* **22**, 3780–3796.
- Wong, M. K.-S. and Woo, N. Y. S. (2006). Rapid changes in renal morphometrics in silver sea bream *Sparus sarba* on exposure to different salinities. *J. Fish Biol.* **69**, 770–782.
- Wund, M. A., Baker, J. A., Clancy, B., Golub, J. L. and Fosterk, S. A. (2008). A test of the “Flexible stem” model of evolution: ancestral plasticity, genetic accommodation, and morphological divergence in the threespine stickleback radiation. *Am. Nat.* **172**, 449–462.
- Yan, J.-J., Chou, M.-Y., Kaneko, T. and Hwang, P.-P. (2007). Gene expression of Na⁺/H⁺ exchanger in zebrafish H⁺-ATPase-rich cells during acclimation to low-Na⁺ and acidic environments. *Am. J. Physiol. Cell Physiol.* **293**, C1814–C1823.
- Yancheva, V., Velcheva, I., Stoyanova, S. and Georgieva, E. (2016). Histological biomarkers in fish as a tool in ecological risk assessment and monitoring programs: a review. *Appl. Ecol. Environ. Res.* **14**, 47–75.